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Avermectin $B_{1}a$ irreversibly blocks postsynaptic potentials at the lobster neuromuscular junction by reducing muscle membrane resistance

(chloride channels/γ-aminobutyric acid/anthelmintic)

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ABSTRACT **Avermectin $B_{1}a$, a macrocyclic lactone with broad spectrum anthelmintic activity, affects neuromuscular transmission in the lobster stretcher muscle. Perfusion of the muscle with 1–10 μg of the drug per ml eliminates inhibitory postsynaptic potentials within a few minutes. Intracellularly recorded excitatory postsynaptic potentials are gradually reduced in amplitude over 20–30 min, and their falling phases become faster; there is no effect, however, on extracellularly recorded excitatory potentials. Avermectin $B_{1}a$ reduces the input resistance of the muscle fibers with a time course similar to that of the reduction of excitatory potentials. Washing for up to 2 hr with drug-free solution fails to reverse the drug's effects. However, perfusion with 20 μg of picrotoxin per ml results in recovery of the excitatory potentials and input resistance. Avermectin $B_{1}a$ also blocks the firing of the crayfish stretch receptor neuron, and this block is also reversed by picrotoxin. We hypothesize that the reduction in excitatory postsynaptic potentials after avermectin $B_{1}a$ treatment is caused solely by reduction in membrane resistance; additional experiments suggest that the reduction in membrane resistance is due to the opening of membrane Cl^{-} channels, perhaps including those regulated by γ-aminobutyric acid at the inhibitory synapse.**

Avermectin $B_{1}a$ (AVM), a macrocyclic lactone derived from mycelia of *Streptomyces avermitilis*, is known to have a potent broad-spectrum anthelmintic activity.[§¶‖] Its molecular structure[**] is shown in Fig. 1. No well-defined physiological effects of this drug have been described. However, the fact that AVM quickly immobilized parasitic nematodes at low doses suggested that its anthelmintic activity may be due to effects on the nematode nervous system (unpublished observation). This prompted us to look for effects of AVM in a number of pharmacologically different synaptic systems. In this paper, we describe the action of AVM on the lobster stretcher neuromuscular junction. The stretcher muscle is innervated by one excitatory and one inhibitory axon (1), but the preparation lacks the presynaptic inhibition (2) seen at some other crustacean neuromuscular junctions. The excitatory transmitter is thought to be glutamate, whereas the inhibitory transmitter is γ-aminobutyric acid (GABA) (3).

MATERIALS AND METHODS

Neuromuscular Preparations. Superficial fibers of the stretcher muscle from the walking leg of the lobster *Homarus americanus* were exposed by chipping away the overlying shell and removing the connective tissue layers. Excitatory postsynaptic potentials (EPSPs) were evoked by stimulation of the nerve bundle in the meropodite segment with hook electrodes or by antidromic stimulation of the opener nerve with a suction electrode. Inhibitory postsynaptic potentials (IPSPs) were evoked by stimulation of the meropodite nerve bundle proximal to a cut placed in the excitatory axon. Preparations were pinned in a Lucite chamber and perfused with lobster saline (468 mM NaCl/10 mM KCl/22 mM $CaCl_2$/8 mM $MgCl_2$/6 mM Tris·HCl, pH 7.2) to which 2% dimethyl sulfoxide (Me_2SO) was added to prevent precipitation of the drug. Control experiments indicated that 2% Me_2SO had no detectable physiological effects. Temperature was maintained at 10–15°C.

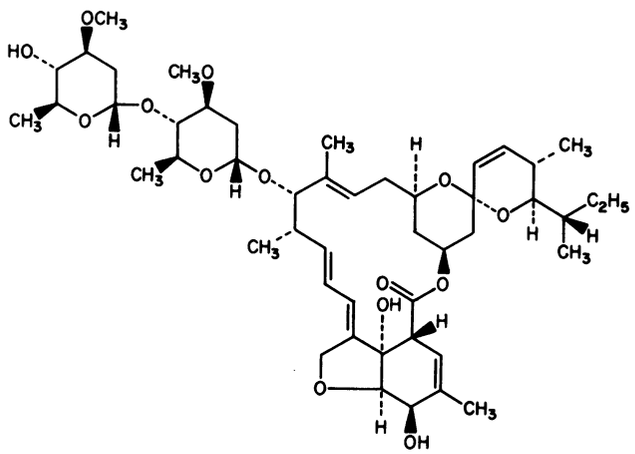

FIG. 1. Molecular structure of AVM.

Muscle preparations from *Ascaris lumbricoides* were maintained in Tyrode's solution under N_2 and studied at 37°C (4). Experiments on the cutaneous pectoris muscle of the frog were performed by published procedures (5).

Crayfish Stretch Receptor Neuron. Stretch receptor neurons with associated muscle elements were dissected from the tail segments of *Procambarus clarkii*. Small pieces of chitin left attached to the muscle elements were held by movable clamps so that small stretches could be applied to the preparation. The dissected specimens were perfused at room temperature with

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Abbreviations: AVM, avermectin $B_{1}a$; GABA, γ-aminobutyric acid; EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential; Me_2SO, dimethyl sulfoxide.

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§ Miller, T. W., *et al.* (1978) *Abstracts of the 18th Interscience Conference on Antimicrobial Agents and Chemotherapy, Oct. 1–4, Atlanta, GA.*

¶ Egerton, J. R., *et al.* (1978) *Abstracts of the 18th Interscience Conference on Antimicrobial Agents and Chemotherapy, Oct. 1–4, Atlanta, GA.*

‖ Burg, R. W., *et al.* (1978) *Abstracts of the 18th Interscience Conference on Antimicrobial Agents and Chemotherapy, Oct. 1–4, Atlanta, GA.*

** Albers-Schönberg, G., *et al.* (1978) *Abstracts of the 18th Interscience Conference on Antimicrobial Agents and Chemotherapy, Oct. 1–4, Atlanta, GA.*

crayfish saline (195 mM NaCl/5.4 mM KCl/13.5 mM $CaCl_2$/2.6 mM $MgCl_2$/6 mM Tris·HCl, pH 7.2) with 2% Me_2SO (6).

Electrical Recording. Intracellular recordings from lobster stretcher muscle fibers and *Ascaris* muscle cells were made with glass micropipettes filled with 3 M KCl or 2 M potassium citrate. Extracellular recordings from lobster synapses were made with micropipettes filled with lobster saline. To measure muscle fiber input resistance, two electrodes were inserted into one fiber within 100 μm of each other; one was used to inject current and the other was used to record the resulting membrane potential displacements. For glutamate iontophoresis, a micropipette filled with 1 M sodium glutamate (pH 8) was positioned at a sensitive site on a muscle fiber. Negative pulses were applied to the glutamate micropipette, and depolarizations of the muscle fiber were recorded by an intracellular KCl-filled micropipette.

Extracellular action potentials were recorded from the static stretch receptor by measuring the voltage across a Vaseline seal through which its axon passed.

Drugs. AVM, prepared by the methods of Miller *et al.*,[§] was obtained from Merck, Sharp & Dohme. It was stored frozen in Me_2SO at −20°C. AVM is insoluble in water but is readily dissolved in Me_2SO. Picrotoxin was obtained from Sigma.

RESULTS

Effect of AVM on Lobster EPSPs and IPSPs. Perfusion of the stretcher muscle with 1–10 μg of AVM per ml caused an irreversible elimination of the IPSP within a few minutes and a more gradual reduction in EPSP amplitude (Fig. 2). Washing the preparation with lobster saline or with 2% Me_2SO/lobster saline for up to 2 hr did not bring about the recovery of IPSPs or EPSPs. Although AVM greatly affected the amplitude of evoked potentials, the muscle membrane potential remained relatively constant. The resting potential of the fibers in control Ringer's solution ranged from −70 to −75 mV, but after AVM treatment a hyperpolarization of up to 5 mV was often observed. This point will be discussed later in regard to the ionic mechanism of the AVM effect. The reduction in EPSP amplitude was also accompanied by a shortening of EPSP duration, its falling phase becoming progressively faster after AVM application (Figs. 2 and 4). The time course of the EPSP falling phase is largely determined by the membrane time constant, which is in turn dependent upon membrane resistance (7). EPSP amplitude is also dependent upon membrane resistance (2, 8). Therefore, a possible explanation for the AVM effects is that the drug reduces the muscle membrane resistance.

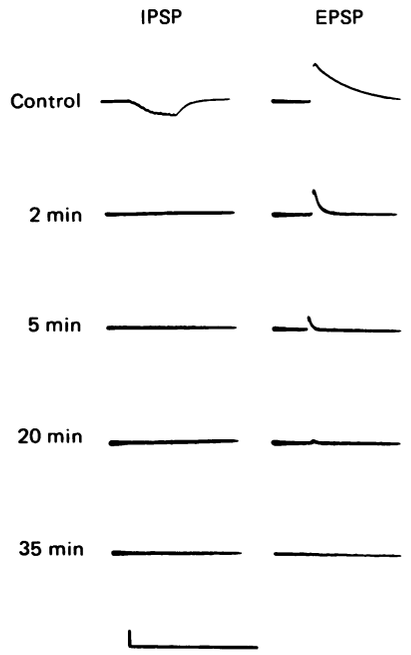

FIG. 2. Effect of AVM on IPSPs and EPSPs in lobster muscle. Records were taken 2, 5, 20, and 35 min after the application of AVM at 1 μg/ml. Trains of IPSPs (stimulation frequency = 30 per sec) were given (left-hand column), and single EPSPs were evoked (right-hand column). Calibration: IPSP, 0.5 mV, 1 sec; EPSP, 2 mV, 200 msec.

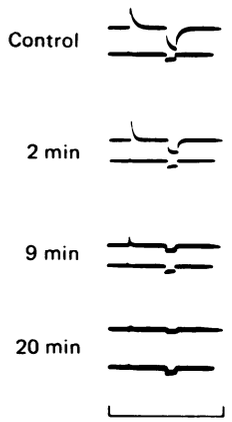

FIG. 3. Effect of AVM on EPSP and on muscle fiber input resistance. Records were taken 2, 9, and 20 min after the application of AVM at 10 μg/ml. Upper traces record a single EPSP (left) and the voltage response to an intracellular current pulse (right). Calibration: 2 mV, 1 sec. Lower traces record intracellularly injected current. (Note: The deflection of the current trace simultaneous with the EPSP is an artifact.) Calibration: 50 nA, 1 sec.

To test this hypothesis more directly, fiber input resistance was measured during application of the drug. The actual membrane resistance of a muscle fiber of finite length is a function of both the input resistance and the fiber length constant (9, 10). However, changes in input resistance alone are a qualitative measure of changes in membrane resistance and are adequate for the present purposes of our study. Fig. 3 shows EPSPs and voltage responses to square current pulses at various times after AVM addition. Input resistance is computed as $\Delta V/\Delta I$. As the EPSP became smaller and faster, the response to the current pulse became smaller and squarer as well. This result demonstrates that, when AVM is reducing the EPSP amplitude and duration, it is also directly reducing membrane resistance. (For a thorough discussion of electrical properties of muscle fibers, see ref. 11). In the experiment illustrated in Fig. 3, the input resistance fell from a control level of 190 kΩ to 20 kΩ after 23 min of AVM treatment. Collected results from six such experiments are presented in Table 1. These results suggest that AVM's effects on EPSPs may be an indirect result of the resistance changes.

Table 1. Reduction of input resistance by AVM

Exp.	Control input resistance, kΩ	Input resistance after AVM treatment	
		T, min	R_i, kΩ
1	144	20	14
2	133	30	13
3	190	23	20
4	150	50	17
5	63	31	17
6	143	24	31

Input resistance (R_i) is measured by passing current into a muscle fiber and recording the voltage change at a nearby point. R_i is defined as the ratio of the voltage change to the current passed. T is the time in min after starting perfusion with AVM.

To demonstrate that the effect of AVM on EPSP can be accounted for by its effect on membrane resistance, we simultaneously recorded intracellular and extracellular EPSPs from the same muscle fibers during drug treatment. The extracellular EPSP is a measure of synaptic current flow, and thus depends on the activity of the presynaptic nerve and on the subsequent transmitter–receptor interaction, but is largely independent of passive muscle membrane properties (12, 13). Fig. 4 shows that the amplitude and time course of the extracellular EPSP remained essentially unchanged during AVM treatment, whereas EPSPs recorded intracellularly exhibited the standard decreases. (As a measure of EPSP time course, we have used $\tau_{1/2}$, defined as the time it takes for EPSP to fall from its peak to one-half of the peak. For extracellular recording the peak is negative; for intracellular recording it is positive.) This strongly suggests that the drug is affecting neither the presynaptic excitatory nerve nor the receptors for the excitatory transmitter. The faster falling phase and decreased amplitude of intracellular EPSP (Fig. 4) caused by AVM are therefore most probably due to a postsynaptic effect that is unrelated to the activity of the synaptic glutamate receptors. These results thus provide additional support for the hypothesis that AVM's effect on the EPSP is caused by reduction of muscle membrane resistance.

If the direct effect of AVM is to reduce membrane resistance, then the intracellular response to iontophoretically applied glutamate should also be reduced by the drug. This was tested directly by applying glutamate iontophoretically to sensitive spots on the muscle fibers and measuring the intracellular response at various times after drug treatment (Fig. 5). The amplitude of the response to an iontophoretic pulse fell with time after AVM application, and the time course of its decline was similar to that for EPSP amplitude. Since iontophoretically applied glutamate interacts directly with postsynaptic receptors to produce an electrical response, the presence of an effect of

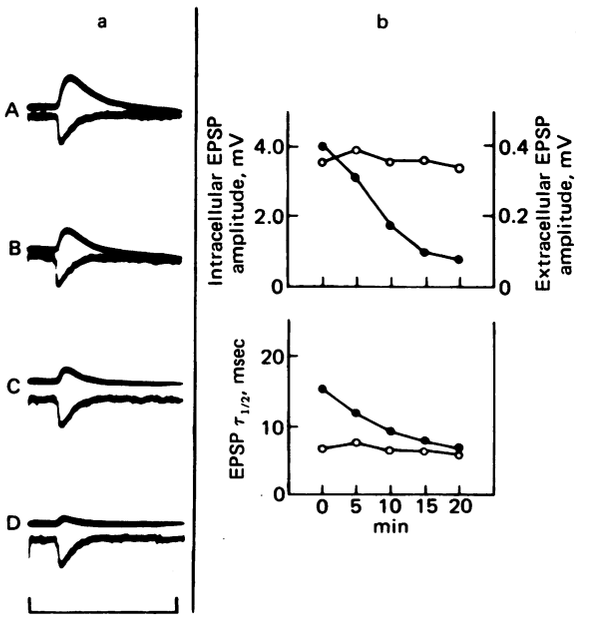

FIG. 4. Effect of AVM on intracellular and extracellular EPSPs. AVM at 5 μg/ml was applied at time = 0. (*a*) A, Control. After application of AVM: B, 5 min; C, 10 min; D, 20 min. Upper traces record intracellular EPSP. Calibration: 2 mV, 100 msec. Lower traces record extracellular EPSP. Calibration: 200 μV, 100 msec. (*b*) Upper graph, EPSP amplitude vs. time after AVM application. Lower graph, $\tau_{1/2}$ of EPSP vs. time after AVM application. $\tau_{1/2}$, Time it takes for EPSP to fall from its peak to one-half of its peak. ●, Intracellular EPSP; ○, extracellular EPSP.

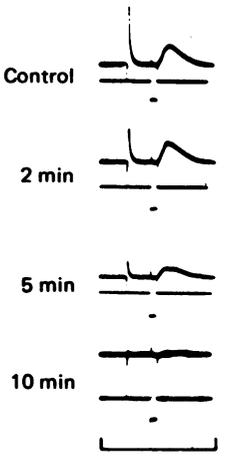

FIG. 5. Effect of AVM on EPSP and iontophoretically applied glutamate. Records were taken 2, 5, and 10 min after application of AVM at 4 μg/ml. Upper traces record EPSP (left) and response to an iontophoretic pulse of glutamate (right). Calibration: 0.5 mV, 1 sec. Lower traces record the iontophoretic current. Calibration: 100 nA, 1 sec.

AVM on that response is further evidence for a postsynaptic action of the drug.

Ionic Dependence of the AVM Effect. It has already been noted that, whereas application of AVM markedly affects muscle input resistance, the drug produces only small changes in resting potential. This indicates that the drug acts to increase the permeability to ions whose equilibrium potentials are close to the resting potential. Cl^- is such an ion, as can be inferred from the facts that the lobster IPSP is caused by an increase in Cl^- conductance and that the equilibrium potential for the IPSP is normally a few millivolts more negative than the resting potential (14). K^+ is also near equilibrium at rest. This is clear from the fact that the muscle membrane acts as a nearly perfect K^+ electrode at the standard K^+ concentration (10 mM) of lobster Ringer's solution (15, 16), and thus K^+ is the major determinant of resting potential.

When AVM was applied in standard lobster Ringer's solution, the muscle membrane hyperpolarized by up to 5 mV within

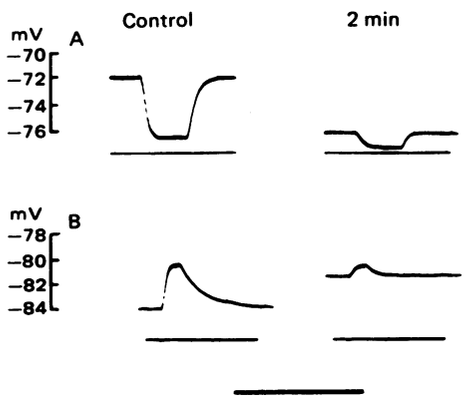

FIG. 6. Effect of AVM on membrane potential and IPSP in standard and K^+-free Ringer's solution. Responses to stimulation of the inhibitory nerve at 100 stimuli per sec before AVM application (control) and after 2 min of AVM (10 μg/ml) treatment. (*A*) Standard Ringer's solution; (*B*) K^+-free Ringer's solution. Mg^{2+} was omitted from both Ringer's solutions to increase IPSP amplitude. The line underneath each record is a voltage level reference used to align the traces in *A* and *B*. The central bar at the bottom of the figure is a time calibration; *A*, 1 sec; *B*, 2 sec.

a few minutes, and the IPSP became greatly reduced (Fig. 6A). This indicates that the drug increases the permeability to ions whose equilibrium potential is somewhat more negative than the control resting potential. To show that the permeability increase is to Cl^- and not K^+, AVM was applied to muscles bathed in K^+-free Ringer's solution. In K^+-free Ringer's solution, the resting potential is more negative than the IPSP equilibrium potential (and thus more negative than the Cl^- equilibrium potential) so that the IPSPs become depolarizing (14). The K^+ equilibrium potential in such a medium must still be more negative than the resting potential, however. Addition of AVM to muscles in K^+-free Ringer's solution caused a depolarization of the membrane by several millivolts, and the IPSPs again became greatly reduced (Fig. 6B). These results, showing that AVM causes hyperpolarization in standard Ringer's solution but depolarization in K^+-free Ringer's solution, suggest that AVM indeed increases the permeability to Cl^- and not K^+. The related observation that the membrane potential change induced by AVM was of the same sign as the Cl^--dependent IPSP in standard as well as K^+-free Ringer's solution provides further support for this hypothesis. The data, however, cannot totally eliminate the possibility that a small increase in K^+ conductance may also occur. The shift in membrane potential toward the IPSP equilibrium potential also helps to explain AVM's more rapid effect on IPSP compared to EPSP. The IPSP became smaller because of both decreased ionic driving force and decreased muscle input resistance, whereas the slower decrease in EPSP was caused solely by the decreased input resistance.

Effect of Picrotoxin on Lobster Muscle Treated with AVM. It is well known that GABA receptors regulate the opening of Cl^- channels that mediate synaptic inhibition in crustacean muscle (14) and that these channels are blocked by picrotoxin (17, 18). If these ionic channels mediate the AVM effects, then picrotoxin might be expected to reverse those effects. To test this possibility, picrotoxin at 20 μg/ml was applied after AVM treatment (Fig. 7). AVM caused the familiar decrease in amplitudes of EPSP and input resistance, but they were greatly increased after picrotoxin application. The added picrotoxin was then washed out with 2% Me_2SO/lobster saline, and the EPSP and input resistance again decreased to low values. These results implicate picrotoxin-sensitive ionic channels in AVM action and further emphasize the irreversible nature of the AVM effect.

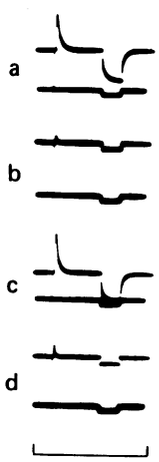

FIG. 7. Effect of picrotoxin on EPSP and on muscle fiber input resistance after AVM treatment. AVM was applied for 25 min. Picrotoxin was then applied for 8 min. The preparation was then perfused with Me_2SO/lobster saline for 27 min. Traces: a, control; b, 24 min after application of AVM at 10 μg/ml; c, 2 min after addition of picrotoxin at 20 μg/ml; d, 25 min after washing with Me_2SO/lobster saline. Upper traces record EPSP (left) and response to intracellular current pulse (right). Calibration: 2 mV, 1 sec. Lower traces record intracellular current pulse. Calibration: 100 nA, 1 sec.

If AVM is affecting GABA receptor–ionophore complexes in the lobster muscle membrane, the drug should also have effects on other crustacean systems that are GABA sensitive. The static crayfish stretch receptor is such a preparation; its dendrites receive inhibitory GABA synapses that are capable of blocking action potential initiation in the cell (6, 19). When the stretch receptor is perfused with AVM at 5 μg/ml, action potentials are blocked so that even a vigorous stretch elicits no firing. Picrotoxin at 60 μg/ml reverses the AVM block, in accordance with the suggestion that AVM acts on the GABA receptor–ionophore complex. The AVM effect on the stretch receptor is in contrast to its apparent lack of effect on the presynaptic excitatory motor axon of the lobster.

Absence of Effects in Other Muscle Preparations. Although AVM could irreversibly immobilize the nematode *Ascaris*, the drug had no effect on the resting potential of its muscle cells and no effect on the tension of the dorsal and ventral muscle strips. *Ascaris* muscle cells receive both excitatory and inhibitory synapses; the transmitters are thought to be, respectively, acetylcholine and GABA based on the effects observed when these compounds are applied to the muscle (20, 21). AVM had no effect on acetylcholine-induced contraction or GABA-induced relaxation of the muscle strips; thus, the ability of AVM to immobilize *Ascaris* is apparently not due to direct effects at the neuromuscular junction (see *Discussion*).

AVM, at concentrations up to 10 μg/ml, had no effect on neuromuscular transmission in the frog cutaneous pectoris muscle, in which acetylcholine is the transmitter (5). At higher doses (>20 μg/ml), a reversible, Ca^{2+}-dependent potentiation of the end-plate potential amplitude and increase in the frequency of miniature end-plate potentials were observed. These results are in sharp contrast to those obtained with the lobster neuromuscular junction and may not share the same basic mechanism of action with the latter.

DISCUSSION

AVM irreversibly blocks IPSPs and reduces EPSPs in the lobster stretcher muscle. The reduction in the amplitude and the increase in the rate of repolarization of the EPSP can be accounted for by the reduction in muscle membrane resistance caused by AVM. The resistance change appears to be the result of increased Cl^- conductance. Extracellular recording demonstrated that the drug had no effect on either transmitter release from the excitatory nerve or the receptors for excitatory transmitter.

The ability of picrotoxin, a well known GABA antagonist (17, 18), to reverse AVM's effects on the EPSP and input resistance suggests that AVM acts on the synaptic GABA system. It must be noted, however, that picrotoxin is not totally specific for GABA receptor Cl^- channels but can also block other Cl^- and K^+ channels in some arthropod systems (22, 23). Nonsynaptic Cl^- channels may also exist in the lobster muscle membrane, and such channels have been documented in locust muscle (22). There are, however, two further indications that synaptic GABA-sensitive Cl^- channels are in fact involved in AVM action. First, AVM affects lobster muscle fibers and the crayfish stretch receptor but not the presynaptic excitatory nerve terminal in the lobster stretcher muscle. One difference among these preparations is that the muscle and stretch receptor both receive GABA synapses (1, 14, 19), whereas the motor nerve does not (6). This could account for the differential AVM effect.

Secondly, binding studies using rat and dog brain synaptosomes show that the highest specific binding of AVM is in the cerebellum, which also has the highest specific binding of GABA and muscimol, a GABA agonist (24), and has a high density of inhibitory GABA synapses (S. S. Pong and C. C. Wang, unpublished results).

It is also interesting that AVM does not block GABA effects on *Ascaris* muscle strips, although it clearly blocks GABA-mediated IPSPs in lobster muscle. The pharmacological properties of the GABA receptor complexes in the two different preparations may be different. There is precedent for this possibility, because, even between the lobster and the closely related crayfish, differences in the relative permeability of the GABA channel to foreign anions have been documented (10, 14). Furthermore, preliminary studies indicate that the GABA-induced relaxation of *Ascaris* muscle strips is not blocked by picrotoxin (unpublished observation), suggesting that the GABA systems in lobster and *Ascaris* are in fact pharmacologically distinct. Since AVM has no effect on either acetylcholine or GABA responses in *Ascaris* muscle, the drug's paralytic action must be exerted at some other site(s) in the nervous system of the nematode. This subject will be dealt with in another publication.

While AVM may act directly on postsynaptic chloride channels, other possibilities exist. It is possible, for instance, that AVM is an ionophore itself. The observation that AVM blocks activity in the crayfish stretch receptor but not in the excitatory nerve argues against this idea, however, because an ionophore would probably be expected to affect both of these preparations. Furthermore, our studies have indicated that AVM has no ionophorous activity on erythrocytes (unpublished observation). Another hypothesis is that AVM causes the release of GABA from inhibitory nerve endings, thus lowering muscle fiber resistance. If this is true, then the GABA release must be sustained for long periods of time without showing fatigue in order to account for the seemingly irreversible nature of the AVM effects. The presence of tonic GABA release in crab muscle has been observed (25). It is conceivable that AVM may greatly accelerate this type of GABA efflux. This possibility is supported by a recent study in which potent stimulation of sustained GABA release from rat brain synaptosomes by AVM was observed (S. S. Pong and C. C. Wang, unpublished data). Tonic release of neurotransmitter has also been observed at the cholinergic vertebrate neuromuscular junction (26, 27).

The fact that the AVM effects cannot be reversed by washing suggests two possibilities regarding its action. The drug could cause its permanent effects by chemically altering some membrane component, but not itself be bound permanently, or it could be irreversibly bound to a membrane component. We cannot yet distinguish between these two possibilities in the lobster, but specific binding to dog brain synaptosomes has been documented. The binding properties of AVM raise the possibility that this drug may be useful in isolating the membrane component that mediates its pharmacological effects.

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